

8. WHAT IS CLAIMED IS:

1. A complex comprising at least one ubiquitin or a derivative thereof, and a protein; wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof; said complex is formed via N-end rule ubiquitylation; and said complex has a specific activity that is at least five-fold greater than the specific activity of in cell lysates, cells or tissues.

2. An *in vitro* complex comprising at least one ubiquitin or a derivative thereof, and a protein; wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof; and said complex is formed via N-end rule ubiquitylation.

3. An isolated complex comprising at least one ubiquitin or a derivative thereof, and a protein; wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof; and said complex is formed via N-end rule ubiquitylation.

4. The complex of any one of claim 1-3, wherein said complex is immobilized on a support and/or linked to a label.

5. A method for producing a complex comprising at least one ubiquitin or a derivative thereof, and a protein; wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, comprising:
- a) forming a mixture comprising a vector containing a clone coding for said protein, an *in vitro* transcription/translation system, an N-rule ubiquitylation system and, optionally, a proteasome inhibitor; and
 - b) incubating said mixture to allow production of said complex.
6. The method of claim 5, further comprising:
- c) isolating said complex.
7. The method of claim 6, wherein said isolating is done by binding to an antibody specific to a poly-ubiquitin chain.
8. The method of claim 6, wherein said isolating is done by binding to an antibody specific for said protein.
9. An activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof; wherein said activated fragment has an activity as a substrate for ubiquitylation that is at least five-fold greater than the equivalent amount of the starting protein.

10. An isolated activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof.

11. An isolated activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, wherein the concentration of said activated fragment is at least two-fold greater than the concentration of activated fragment from a natural source.

12. The activated fragment of claim 9, 10 or 11, wherein said activated fragment is immobilized on a support and/or linked to a label.

13. The activated fragment of claim 9, 10, 11 or 12, wherein said activated fragment is further subjected to a proteolysis which removes a C-terminal portion of said activated fragment.

14. A method of producing an activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, comprising:

- a) forming a mixture comprising a vector containing a clone coding for said protein, an *in vitro* transcription/translation system that comprises a protease capable of generating said activated fragment, an inhibitor of N-rule ubiquitylation and an inhibitor of proteasome-mediated degradation; and
- b) incubating said mixture to allow production of said activated fragment.

15. A method of producing an activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMG13, HSPC144 and CDC6 and fragments and derivatives thereof, comprising:

- a) forming a mixture comprising a complex of claim 1, 2 or 3 and a deubiquitylating system; and
- b) incubating said mixture to allow production of said activated fragment.

16. The method of claim 14 or 15, further comprising:

- c) isolating said activated fragment.

17. An assay composition comprising an ubiquitin and a protein, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMG13, HSPC144 and CDC6 and fragments and derivatives thereof and said ubiquitin and/or said protein is immobilized on a support and/or linked to a label.

18. A method for identifying a protease cleavage site which is cleaved by a protease to expose an N-degron in a protein, comprising:
- a) forming a mixture comprising said protein, and said protease;
 - b) incubating said mixture to allow the protease to cleave at a cleavage site;
 - and
 - c) analyzing cleavage products to determine the location of the cleavage site.
19. The method of claim 18, wherein said location of the cleavage site in said protein is determined by gel electrophoresis.
20. The method of claim 18, wherein said location of the cleavage site in said protein is determined by chromatography.
21. The method of claim 18, wherein said protein is radioactively labeled.
22. The method of claim 18, wherein said location of the cleavage site in said protein is determined by peptide sequencing.
23. The method of claim 18, wherein said location of the cleavage site is determined by mass-spectroscopy.
24. The method of claim 18, further comprising:

- d) forming a mixture comprising said protein, said protease and an N-rule ubiquitylation system and a proteasome inhibitor;
- e) incubating said mixture to allow the protease to cleave at a said cleavage site and the ubiquitylation system to ubiquitylate at exposed N-degrons; and
- f) comparing the products of steps b) and e) to identify protease products ubiquitylated by the N-end rule ubiquitylation system.

25. A method for identifying at least one protease which cleaves a protein to expose an N-degron, the protein having a pro-N-degron and being selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, comprising:

- a) forming a mixture comprising said protein; and
- b) screening a protease library for proteases which bind said protein.

26. The method of claim 24, further comprising the step of:

- c) identifying proteases which expose said N-degron.

27. A method for identifying at least one protease which cleaves a protein to expose an N-degron, the protein having a pro-N-degron and being selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, comprising:

- a) forming a mixture comprising said protein and a putative protease; and

- b) determining if said protease cleaves said protein to expose an N-degron.
28. A method for identifying at least one E3 ligase, comprising:
- a) combining a putative E3 ligase with an N-end rule substrate; and
 - b) measuring binding of the putative E3 ligase to said N-end rule substrate.
29. A method for identifying at least one E3 ligase, comprising:
- a) combining a putative E3 ligase with an N-end rule substrate and ubiquitylation system; and
 - b) measuring ubiquitylation and/or Ub-dependent degradation of said N-end rule substrate.
30. The method of claim 29, wherein said combining a putative E3 ligase with an N-end rule substrate and ubiquitylation system is done in a presence of at least one proteasome inhibitor.
31. The method of claim 29, wherein said ubiquitylation system lacks an active E3 ligase.
32. The method of claim 29, wherein the activity of the putative E3 ligase is greater than the activity of endogenous E3 ligases in said ubiquitylation system by at least two fold.

33. The method of claim 29, wherein said combining a putative E3 ligase with an N-end rule substrate and ubiquitylation system is carried out in the presence of inhibitors of endogenous E3 ligase activity.

34. The method of claim 28 or 29, wherein said N-end rule substrate comprises an activated N-end rule substrate having an exposed N-degron.

35. A method for identifying one or more active compounds that modulate N-end rule dependent ubiquitylation of a protein selected from the group comprising aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6, comprising:

- a) forming a mixture comprising said protein or a fragment or derivative thereof, an N-rule ubiquitylation system, one or more candidate compounds and, optionally, a proteosome system;
- b) measuring N-end rule ubiquitylation and/or proteosome-mediated degradation of said compound; and
- c) identifying one or more compounds that modulate the rate of ubiquitylation or degradation.

36. The method of claim 35, wherein said protein includes a pro-N-degron and said mixture of step a) further includes a protease which exposes said N-degron.

37. A method for identifying one or more active compounds that modulate N-end rule dependent ubiquitylation of a protein selected from the group comprising aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6, comprising:

- a) forming a mixture comprising an activated fragment of said protein having an exposed N-degron, an N-rule ubiquitylation system, one or more candidate compounds, and optionally a proteosome system;
- b) detecting N-end rule ubiquitylation and/or proteosome-mediated degradation of said activated fragment; and
- c) identifying one or more compounds that modulate rate of ubiquitylation or degradation.

38. The method of claim 35, 36 or 37, wherein said active compound modulates activity of an E1 ligase, E2 ligase, E3 ligase, a protease that exposes said N-degron, or a combination thereof.

39. The method of claim 37, wherein said active compound modulates activity of an E1 ligase, E2 ligase and/or E3 ligase, or a combination thereof.

40. A method for determining the mechanism of a compound that affects N-end rule ubiquitylation, comprising:

- a) performing the identifying method of claim 36;
- b) repeating said identifying method, except that said mixture further comprises an inhibitor of N-end rule ubiquitylation or said protein is

replaced with a pre-activated fragment of said protein having said exposed N-degron; and

- c) determining whether said compound is specific for said protease, and/or said N-end rule ubiquitylation system.

41. A method for determining the mechanism of a compound that affects N-end rule ubiquitylation, comprising:

- a) performing the identifying method of claim 35;
- b) repeating said identifying method, except that said mixture further comprises an additional modulator of Type I, Type II and/or Type III N-end rule ubiquitylation; and
- c) determining where said compound affects Type I, Type II and/or Type III N-end rule ubiquitylation.

42. A method of making a pharmaceutical formulation containing one or more active compounds which modulate N-end rule ubiquitylation of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMG3, HSPC144 and CDC6, comprising:

- a) forming a mixture comprising said protein, or an activated fragment of said protein having an exposed N-degron, an N-rule ubiquitylation system, one or more candidate compounds and, optionally, a proteosome system;
- b) detecting N-end rule ubiquitylation and/or proteosome-mediated degradation of said protein;

- c) identifying one or more active compounds from said one or more candidate compounds; and
- d) incorporating at least one of said one or more active compounds into a pharmaceutical formulation comprising said at least one active compound and suitable carrier.

43. The method as in any one of claims 35-42, wherein said one or more active compounds are inhibitors of N-end rule ubiquitylation.

44. The method as in any one of claims 35-42, wherein said one or more active compounds are promoters of N-end rule ubiquitylation.

45. The method as in any one of claims 35-42, wherein said one or more active compounds are naturally occurring.

46. The method as in any one of claims 35-42, wherein said one or more candidate compounds are selected from a compound library.

47. The method as in any one of claims 35-42, wherein said one or more candidate compounds are selected from a compound library of FDA approved drugs.

48. A method for modulating N-end rule ubiquitylation of a protein comprising administering one or more active compounds as in any one of claims 35-42.

49. A method for changing the level of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 (*in vitro*, *in vivo* or *ex vivo*), comprising administering an effective amount of an active compound that modulates the rate of N-end rule ubiquitylation of said protein.

50. A method of creating a modified protein by modifying a protein of interest to change its susceptibility to N-end rule degradation, said protein of interest selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6, comprising:

- i) decreasing the susceptibility of the protein to N-end rule degradation by modifying a protease cleavage site to prevent protease cleavage at said site, wherein cleavage at said protease cleavage site leads to exposure of an N-degron;
- ii) increasing the susceptibility of the protein to N-end rule degradation by introducing a protease cleavage site for a known protease, wherein cleavage at said protease cleavage site leads to exposure of an N-degron;
or
- iii) decreasing the susceptibility of the protein to N-end rule degradation by modifying a protease cleavage site that when cleaved exposes an N-degron so that, after modification, the C-terminal product of said protease cleavage is not recognized by an N-end rule E3 ligase.

51. The method of claim 50, further comprising expressing said modified protein in a cell.
52. The method of claim 51, wherein the cell does not express the protein of interest.
53. A method of generating a phenotypic cell line or animal, comprising:
- a) generating a clone coding for a mutated form of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, the mutated protein having a mutated protease cleavage site and/or N-degron and thereby modulating the susceptibility of the protein to N-end rule ubiquitylation; and
 - b) using said vector to transfect said cell line or to generate a transgenic animal by homologous or non-homologous recombination; and
 - c) detecting at least one phenotypic change relative to a control cell line or animal expressing the non-mutated form of the protein.
54. A kit for producing an N-end rule ubiquitylated protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof comprising, in two or more containers, one or more DNA sequences coding for the protein and one or more assay components selected from the group consisting of: (a) *in vitro* transcription and optionally translation system; (b) a plate; (c) one or more binding reagents; (d) a pH buffer; (e) one or more

blocking reagents; (f) one or more antibodies; (g) luminescent label; (h) luminescence co-reactant; (i) preservatives; (j) stabilizing agents; (k) enzymes; (l) detergents; (m) inhibitors and (n) desiccants.

55. A library of N-end rule ubiquitylation substrates including at least two proteins, or fragments or derivatives thereof, selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6.

56. A method of producing an activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, comprising:

- a) forming a mixture comprising said protein and a protease that cleaves said protein to form said activated fragment; and
- b) incubating said mixture to allow production of said activated fragment.